# SUPERCRITICAL FLUID EXTRACTION OF BIOACTIVE COMPOUNDS FROM WALNUT LEAVES

***[1]Popovici Cristina, [2]Gîtin Liliana, [2]Alexe Petru**

[1]Technical University of Moldova – Chişinău, Moldova
[2]"Dunarea de Jos" University – Galați, Romania

*** Popovici Cristina, popovici.kristina@gmail.com**

**Abstract:** The extractive efficiency of bioactive compounds from plant material is greatly depended on the extraction techniques. In this study, supercritical fluid extraction (SFE) of bioactive compounds from walnut leaves was used. Work conditions of supercritical fluid extraction were pressure level at 200 bar, temperature at 50 $^0$C and flow rate of $CO_2$ - 20 kg/h. Efficiency of the SFE was evaluated by measuring total polyphenol content (Folin-Ciocalteu assay), reaction kinetics and reducing power of the extract towards DPPH$^\cdot$ free radical. Walnut leaves extract obtained by supercritical fluid extraction showed the good antioxidant properties and could be used as a natural, low cost source of bioactive compounds.
**Keywords:** walnut leaves, supercritical fluid extraction, bioactive compounds, Folin-Ciocalteu's and DPPH$^\cdot$ assays

## 1. Introduction

Walnut leaves are considered a source of healthcare compounds, and have been widely used in traditional medicine [5]. In some European countries, especially in rural areas, dry walnut leaves are frequently used to prepare infusions for their antiradical and antibacterial properties [1, 8].

In walnut leaves, naphtoquinones and flavonoids are considered as major phenolic compounds [12]. Juglone (5-hydroxy-1,4-naphthoquinone) is known as being the characteristic compound of *Juglans* spp. and is reported to occur in fresh walnut leaves [4, 6, 7, 11, 12]. Nevertheless, because of polymerization phenomena, juglone only occurs in dry leaves at vestigial amounts [12]. Several hydroxycinnamic acids (3-caffeoylquinic, 3-p-coumaroyquinic and 4-p-coumaroylquinic acids) and flavonoids (quercetin 3-galactoside, quercetin 3-arabinoside, quercetin 3-xyloside, quercetin 3-rhamnoside and two other partially identified quercetin 3-pentoside and kaempferol 3-pentoside derivatives) of defferent walnut cultivars collected at different times were studied [1]. In addition, the existence of 5-caffeoylquinic acid was also reported [12].

Supercritical fluid extraction with $CO_2$ is an environmentally benign and efficient extraction technique for solid materials and has been studied for the separation of bioactive compounds from plant materials. Organic solvent-free extract can be obtained and the low operating temperature makes it possible to preserve all natural properties of plant materials [2].

In this paper, the extraction of bioactive compounds from walnut leaves was effectuated by applying supercritical fluid extraction. This method has been proven to be desirable for phenolics extraction and has many advantages such as increasing extraction yield, shortening extraction duration. Evaluation of the efficiency of supercritical fluid extraction process application was measured by total polyphenol content and antioxidant activity assays.

## 2. Materials and methods

*2.1. Plant material*

Walnut (*Juglans regia* L.) leaves were harvested during Autumn, October 2011, in Chisinau, Central Moldova. Fresh and healthy leaves were manually collected from the middle third of branches exposed to sunlight. The leaves were dried at room temperature, powdered and packed in paper bags in order to protect them from light. Voucher specimens were transported to the laboratory of Faculty of Food Science and Engineering, University Dunarea de Jos Galati, Romania.

*2.2. Chemical and reagents*

1,1-Diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) as free radical form (95%), Folin-Ciocalteu's phenolic reagent, sodium carbonate were supplied by Sigma-Aldrich. 3,4,5-trihydroxybenzoic acid were obtained from Alfa Aesar. Methanol (99,8%) and ethanol (96%) were provided by Eco-Chimie (Chisinau, Moldova). Carbon dioxide gas (99.92%) supplied by Technic Gaz (Buzau, Romania) and delivered in cylinders with siphon tube for feeding with liquid solvent of the extraction plant.

*2.3. Supercritical fluid extraction*

Equipment used for supercritical fluid extraction (SFE) of walnut dried leaves was designed and supplied by Natex Prozesstechnologie GembH (Ternitz, Austria). Extraction parameters used in this work are shown in table 1.

***Table 1.*** Parameters of supercritical fluid extraction process of walnut leaves

| Extract | Pressure [bar] | | | Temperature [$^0$C] | | | Flow rate of $CO_2$ | | Dynamic extraction time [min] |
|---|---|---|---|---|---|---|---|---|---|
| | C 30 | S 40 | S 45 | C 30 | S 40 | S 45 | kg/h | kg (Total) | |
| Walnut leaves | 200±4 | 100±1 | 50±1 | 50,8±0,9 | 36,6±0,2 | 17,4±2 | 20±1 | 81 | 240,067 |

The pressure level was set at 200 bar as is above the critical pressure of the $CO_2$ solvent (73,8 bar), and as suggested by previous workers for extraction of phenolic compounds from plant material. Temperature of 50 $^0$C is above the critical temperature for $CO_2$ (31,06 $^0$C) and this temperature is generally used in the extraction of plant materials by SC-$CO_2$. The selected value of the temperature (50 $^0$C) was low enough to avoid the damage of heat sensitive compounds.

*2.4. Total polyphenol content measurement*

For quantification of total polyphenol content, the Folin-Ciocalteu's method was used [10]. A volume of 0.5 ml of Folin-Ciocalteu's reagent was added to a dark flask, containing 0.5 ml of the each extract sample and 10 ml of distilled water. After 5 min, 8 ml of a 7.5% aqueous sodium carbonate solution was added to the mixture and the content was mixed thoroughly. The samples were kept in dark for 2h and then the absorbance was measured at 765 nm with HACH LANGE DR-5000 UV/vis spectrophotometer. Three parallel samples were analyzed. Gallic acid was used for constructing the standard curve, obtained in advance.Concentration range of gallic acid was of 0.05-0.5 mg/ml. The results of total polyphenol content were expressed as mg of gallic acid equivalents per ml of extract (mg GAE/ml).

*2.5. Determination of DPPH radical scavenging activity*

The antioxidant activity of walnut leaves extracts as well as the kinetics of inhibition of free radicals were studied in terms of radical scavenging ability using the stable DPPH• method [3]. 0.1 ml of the extract sample was added to 3.9 ml of 60 μM solution of DPPH• in methanol. The reaction was carried in dark and the absorbance was recorded at 515 nm to determine the concentration of remaining DPPH•. Methanol as instead of DPPH• solution was used as blank solution. The values of $[DPPH^{\bullet}]_t$ at each reaction time were calculated according to the calibration curve (in the concentration range of 0.38-38 μg/ml): $A_{515\ nm} = 0.0293\ [DPPH^{\bullet}]_t - 0.0072$, where the concentration $[DPPH^{\bullet}]_t$ is expressed in μg/ml. The coefficient of linear correlation of the above relation is R = 0.9999. The radical scavenging activity (RSA) was calculated using the equation [9]:

$$RSA = 100\% \bullet ([DPPH^{\bullet}]_0 - [DPPH^{\bullet}]_{30})/ [DPPH^{\bullet}]_0$$

where $[DPPH^{\bullet}]_0$ is the concentration of the DPPH• solution (without sample) at t=o min and $[DPPH^{\bullet}]_{30}$ is the remained DPPH• concentration at t=30 min. Lower $[DPPH^{\bullet}]_t$ in the reaction mixture indicates higher free radical scavenging activity.

*2.6. Statistical analysis*

Variance analysis of the results was carried out by least square method with application of coefficient Student and Microsoft Office Excel program version 2007. Differences were considered statistically significant if probability was greater than 95% (p-value <0.05). All assays were performed by triplicate at room temperature 20 ± 1 $^0$C. Experimental results are expressed as average ± SD (standard deviation).

## 3. Results and discussion

Walnut leaves are inedible by-products in walnut plantations, which may be a potent source of antioxidants, and have a potential as a value-added ingredients for functional foods.

Several studies on the extraction of bioactive compounds from different cultivars of walnut leaves have been published [1, 8]. Knowledge of the behavior of the factors influencing the process conditions is necessary to enhance the optimization extraction efficient for any bioactive compound. Previous findings have reported the influence of many independent variables, such as extraction method, solvent composition, pH, temperature, pressure and extraction time on the yields of bioactive compounds which can be extracted from diverse natural products. The positive or negative role of each factor in the mass transfer of the process is not always clear; the chemical characteristics of the solvent and the diverse structures and compositions of the natural products mean that each material-solvent system has a different behavior, which cannot be predicted. In this study walnut leaves extract was obtained by supercritical fluid extraction with carbon dioxide as a solvent and showed the polar properties. Chloroform as a solvent was used to analyze the antioxidant potential and content of bioactive compounds analysed extract.

In this study, the UV/Vis spectra of the walnut leaves extracts were analysed in the wavelength range 270 - 710 nm. From identification of bioactive compounds by UV/Vis spectra, it clearly revealed that studied extract contain phenolic acids (237, 245, 270 and 290 nm), flavonoids (333 nm) and carotenoids (417, 457, 484 and 537 nm). The total phenolic content (by Folin-Ciocalteu assay) was 477,59 mg/g in walnut leaves extract. Obtained UV/Vis spectra of walnut leaves extract with concentration of 1 mg/ml is presented in figure 1.

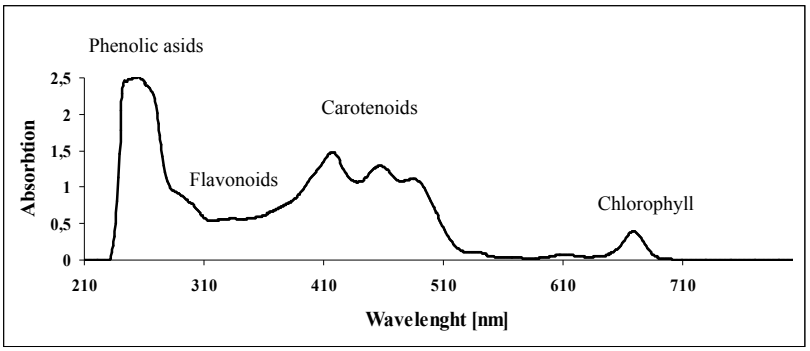


***Fig. 1.*** UV/Vis spectra of walnut leaves extract

The knowledge of the kinetics of atom transfer is important because free radicals in the organism are short-lived species, what implies that the impact of a substance as an antioxidant depends on its fast reactivity towards free radicals. In this study the antioxidant capacity of the walnut leaves extracts were analyzed as the kinetics of inhibition of free radicals (the percentage of DPPH• remaining at steady state). The work concentrations of the walnut leaves extracts were between 0,1 and 10 mg/ml. Reaction kinetics of DPPH· free radical with walnut leaves extracts are shown in figure 2.

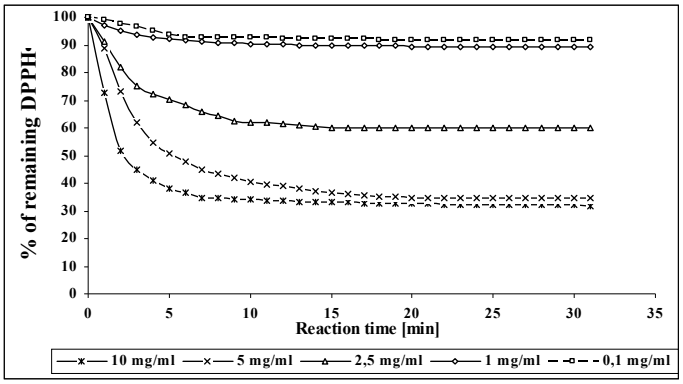


***Fig. 2.*** Reaction kinetics of DPPH· free radical with different concentration of walnut leaves extracts.

It is well known that the absorbance decreases as a result of a colour change from purple to yellow when the radical is scavenged by antioxidants through donation of hydrogen to form the stable DPPH-H molecule. A more rapid decrease of the absorbance means more potent antiradical activity, expressed in terms of hydrogen donating ability of the compounds.

Walnut leaves extract obtained by SFE possess good amounts of bioactive compounds and a significant radical scavenging activity towards stable DPPH free radical (Figure 3).

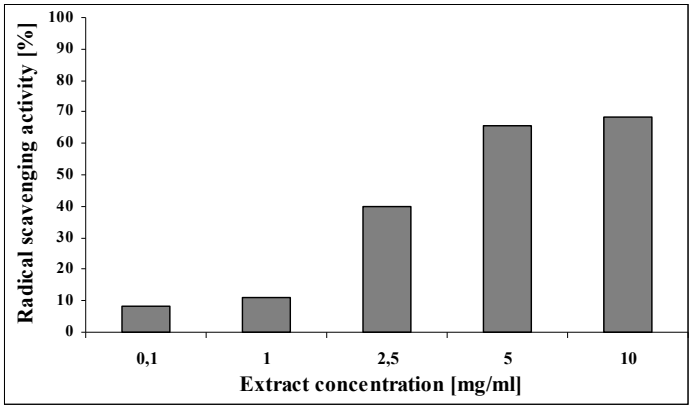


***Fig. 3.*** Scavenging activity of walnut leaves extracts on the DPPH· free radical

The antioxidant activity analyses were performed with the walnut extracts of different concentration in chloroform. The antioxidant activity value of tested extracts was expressed as radical scavenging activity and this parameter was in the range of 8,16 – 68,16%. The amount of extract needed to decrease the initial DPPH• concentration by 50% is usually used for antioxidant activity appreciation of studied extract. In this study $EC_{50}$ for walnut leaves extract was also determined. Thus value was 3,74 mg/ml for walnut leaves extract (Figure 4).

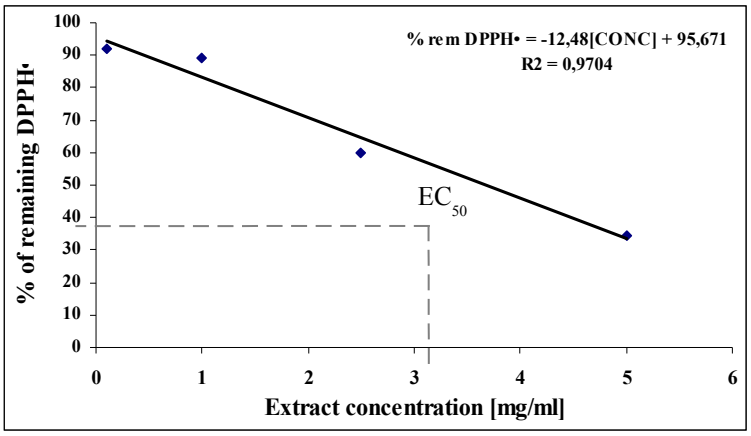


***Fig. 4.*** Reducing power ($EC_{50}$) of the walnut leaves extracts towards DPPH· free radical

## 4. Conclusion

The study suggests the walnut leaves, as a by-product, can become the row material for bioactive compounds extraction. To increase the extraction efficiency, and consequently, reduce the extraction time of bioactive compounds and extraction yields from walnut leaves it can be proposed to increase the polarity of carbon dioxide solvent by addition of small amount of a liqiud co-solvent (modifier). Ethanol is more preferable as a co-solvent in supercritical fluid extraction because of it lower toxicity.

## 5. References


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